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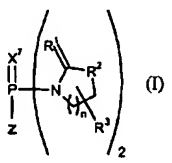
(54) Title: COUPLING REAGENT FOR H-PHOSPHONATE CHEMISTRY



(I)

(57) Abstract: The present invention relates to methods of utilizing compounds of formula (I), wherein X?7¿ is =O or =S; Z is a halogen; R?1¿ is =O or =S; R?2¿ is -O- or -S-; R?3¿ is hydrogen, or a substituent, preferably a halogen, a nitro group, or an alkyl group; and n is 1 or 2 as a coupling reagent to form H-phosphonate diesters and oligonucleotides.

COUPLING REAGENT FOR H-PHOSPHONATE CHEMISTRY

BACKGROUND OF THE INVENTION

The idea that synthetic oligonucleotides or their analogs might find application in chemotherapy has attracted a great deal of attention both in academic and industrial laboratories. Antisense and antigene approaches to chemotherapy have profoundly affected the requirements for synthetic oligonucleotides. Whereas milligram quantities have generally sufficed for molecular biological purposes, one gram to greater than 100 gram quantities are required for clinical trials. Several oligonucleotide analogs that are potential antisense drugs are now in advanced clinical trials. When one of these sequences becomes approved, kilogram or more probably multikilogram quantities of a specific sequence or sequences will be required.

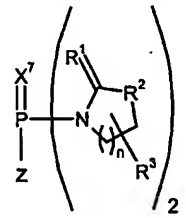
Fast and efficient methods for generating oligonucleotides are in great demand. Three main methods, namely the phosphotriester, phosphoramidite, and H-phosphonate approaches have proven to be effective for the chemical synthesis of oligonucleotides. While the phosphotriester approach has been used most widely for synthesis in solution, the phosphoramidite and H-phosphonate approaches have been used almost exclusively in solid phase synthesis. The phosphoramidite method, while effective, is time consuming because an oxidation step is required after each condensation reaction (also referred to herein as the coupling reaction). The phosphotriester method is also time consuming, as those oligonucleotides that have not had a monomer added to them during the condensation step must be capped in a separate step in order to prevent further chain elongation in future cycles.

The H-phosphonate method for synthesizing oligonucleotides involves condensation of a nucleoside H-phosphonate and a nucleoside or nascent oligonucleotide. This methodology does not require a capping step or oxidation step after each condensation reaction. Generally, the H-phosphonate linkages formed as a result of the condensation reaction can be oxidized once the entire oligonucleotide chain is synthesized. In an alternative H-phosphonate method, the coupling step is performed in the presence of a sulfur transfer reagent which reacts with the H-phosphonate diester formed in the coupling reaction to form, *in situ,* a phosphorothioate triester (see WO99/09041).

Several condensing agents have been shown to successfully promote condensation of H-phosphonates with nascent oligonucleotides. Many of these condensing agents, however, exhibit lower coupling efficiencies and generate side reaction products when used in large-scale synthesis reactions. In order to obtain longer oligonucleotides in good yield and/or to reduce the cost of reagents used to synthesize oligonucleotides, it would be advantageous to improve H-phosphonate methodologies.

SUMMARY OF THE INVENTION

It has been discovered that a compound represented by Structural Formula I:



I.

wherein X^7 is =O or =S; Z is a halogen, preferably -Cl or -Br; R^1 is =O or =S; R^2 is -O- or -S-; R^3 is -H or a substituent, preferably a halogen, a nitro group, or an alkyl group; and n is 1 or 2 will effectively promote condensation of an H-phosphonate, particularly a nucleoside H-phosphonate, and a substrate comprising a free hydroxy group. Preferably, R^1 is =O, R^2 is -O-, R^3 is -H, n is 1 and X^7 is =O. It is most preferred that Z is Cl. Thus, the method of the invention can be used to form oligonucleotides in solution or on a solid support. In addition, the coupling can be done in the presence of a sulfur transfer reagent to form a phosphorothioate triester. The coupling reagent represented by Formula I is suitable for use in the methods taught in PCT publication WO 99/09041, the entire teachings of which are hereby incorporated by reference.

Accordingly, the present invention features a method of preparing an H-phosphonate diester, involving the coupling of an H-phosphonate with a substrate comprising a free hydroxy group in the presence of a compound represented by Structural Formula I. The H-phosphonate diester formed can be protected or deprotected. The H-phosphate linkages formed in the coupling step can be derivatized using a sulfur transfer agent or other oxidizing agent upon completion of the oligonucleotide synthesis. Alternatively, phosphorothioate triester linkages can be formed *in situ* by reacting the H-phosphonate with a sulfur transfer agent. The phosphorothioate triester oligonucleotide is treated with a base upon completion of the oligonucleotide synthesis to form a phosphorothioate diester oligonucleotide or with an oximate and a base to form a phosphodiester oligonucleotide. Any of these methods may be carried out in solution or on a solid support.

A compound represented by Structural Formula I can also be used in coupling reactions to generate oligonucleotides, which can be protected or deprotected, and which can also be derivatized using a sulfur transfer agent or other oxidizing agent. Synthesis of phosphodiester and phosphorothioate triester oligonucleotides can be achieved *in situ* by reacting the H-phosphonate diester with a sulfur transfer reagent. In addition, preparation of oligonucleotides using a compound represented by Structural Formula I can be carried out in solution or on a solid support. The synthetic oligonucleotide preferably has from 2

to about 100 nucleobases. More preferably, the synthetic oligonucleotide has 2 to about 75 nucleobases. Many synthetic oligonucleotides of current therapeutic interest comprise from about 8 to about 40 nucleobases. Oligonucleotides of such lengths can be generated using the methods described herein.

The present invention has several advantages. The methods described herein provide a new coupling procedure for the synthesis of phosphodiesters and oligonucleotides that in many embodiments (a) is extremely efficient and does not lead to side-reactions, (b) proceeds relatively rapidly, and (c) is equally suitable for the preparation of oligonucleotides, their phosphodiester or phosphorothioate analogs, and chimeric oligonucleotides containing both phosphodiester and phosphorothioate diester internucleotide linkages. The use of a compound represented by Structural Formula I in the promotion of H-phosphonate condensation reactions has the advantage of minimizing modification of the O6 group of guanosine and the O4 group of thymidine, which are susceptible to modification with other condensation promoters such as pivaloyl chloride, adamantyl chloride, and diphenyl phosphoryl chloride. In addition, a compound represented by Structural Formula I is less expensive than other condensation promoters, such as adamantyl chloride, and is easily removed after the condensation reaction.

Another advantage of the present invention is that the coupling methods described herein can be used for either solution synthesis or solid support synthesis of oligonucleotides. The advantages of solid support synthesis over solution synthesis are (i) that it is much faster; (ii) that coupling yields are generally higher; (iii) that it is easily automated; and (iv) that it is completely flexible with respect to sequence. Thus, solid phase synthesis is particularly useful if relatively small quantities of a large number of oligonucleotides sequences are required for combinatorial purposes. However, if kilogram quantities of a particular sequence of moderate size are required, speed and flexibility become relatively unimportant, and synthesis in solution is likely to be highly advantageous. Solution synthesis also has the advantage over solid phase synthesis in that block coupling (i.e., the addition of two or more nucleotide residues at a time) is more feasible and scaling-up is unlikely to present a problem. The use of a coupling method involving a compound represented by Structural Formula I provides the flexibility to synthesize oligonucleotides based on the desired criteria.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 represents a preferred coupling reagent N,N-bis-($_2$-oxo-$_3$-oxazolidinyl)-phosphorodiamidic chloride (also referred to as BOP-Cl).

FIG. 2 is a reaction scheme for the activation of a nucleoside-H-phosphonate monomer in the formation of an H-phosphonate diester linkage.

FIG. 3A represents a protected dGC dimer phosphorothioate synthesized using methods described herein.

FIG. 3B represents a protected dGA dimer phosphorothioate synthesized using methods described herein.

FIG. 3C represents a protected dTG dimer phosphorothioate synthesized using methods described herein.

DETAILED DESCRIPTION OF THE INVENTION

The H-phosphonate employed in the methods of the present invention is advantageously a protected nucleoside or oligonucleotide H-phosphonate, or an analog thereof, preferably comprising a 5' or a 3' H-phosphonate function, and more preferably, a 3' H-phosphonate function. Preferred nucleosides are 2'-deoxyribonucleosides and ribonucleosides, for example, 2'-O-(alkyl, alkoxyalkyl, or alkenyl)-ribonucleosides. Preferred oligonucleotides are oligo(2'-deoxyribonucleotides) and oligoribonucleotides, for example, 2'-O-(alkyl, alkyoxyalkyl, or alkenyl)-oligoribonucleotides.

As used herein, "nucleoside" means a molecule that is made of a purine or pyrimidine base linked to sugar. Nucleoside bases include, but are not limited to, naturally occurring bases, such as adenine, guanine, cytosine, thymine, and uracil and modified bases such as 7-deazaguanine, 7-deaza-8-azaguanine, 5-propynylcytosine, 5-propynyluracil, 7-deazaadenine, 7-deaza-8-azaadenine, 7-deaza-6-oxopurine, 6-oxopurine, 3-deazaadenosine, 2-oxo-5-methylpyrimidine, 2-oxo-4-methylthio-5-methylpyrimidine, 2-thiocarbonyl-4-oxo-5-methylpyrimidine, 4-oxo-5-methylpyrimidine, 2-amino-purine, 5-fluorouracil, 2,6-diaminopurine, 8-aminopurine, 4-triazolo-5-methylthymine, 4-triazolo-5-methyluracil and hypoxanthine.

The term "oligonucleotide," as used herein, includes naturally occurring oligonucleotides, for example, 2'-deoxyribonucleic acids (hereinafter "DNA") and ribonucleic acids (hereinafter "RNA") and nucleic acids containing modified sugar moieties, modified phosphate moieties, or modified nucleobases. Modification to the sugar moiety includes replacing the ribose ring with a hexose, cyclopentyl, or cyclohexyl ring. Alternatively, the D-ribose ring of a naturally occurring nucleic acid can be replaced with an L-ribose ring, or the b-anomer of a naturally occurring nucleic acid can be replaced with the a-anomer. The oligonucleotide may also comprise one or more abasic moieties. Modified phosphate moieties include phosphorothioates, phosphorodithioates, methyl phosphonates, methyl phosphates, and phosphoramidates. Such nucleic acid analogs are known to those of skill in the art.

When the H-phosphonate is a protected deoxyribonucleoside, ribonucleoside, oligodeoxyribonucleotide, or oligoribonucleotide derivative comprising a 3' H-phosphonate function, the 5' hydroxy function is advantageously protected by a suitable protecting group. Examples of such suitable protecting groups include acid labile protecting groups, particularly trityl and substituted trityl groups, such as dimethoxytrityl and 9-phenylxanthen-9-yl groups; and base labile-protecting groups, such as FMOC.

As used herein, an "acid labile protecting group" is a protecting group that can be removed by contacting the group with a Bronsted or a Lewis acid. Acid labile protecting groups are known to those skilled in the art. Examples of common acid labile protecting groups include substituted or unsubstituted trityl groups (Greene *et al.*, *Protective Groups in Organic Synthesis* (1991), John Wiley & Sons, Inc., pages 60-62), substituted or unsubstituted tetrahydropyranyl groups (*Id.*, pages 31-34), substituted or unsubstituted tetrahydrofuranyl groups (*Id.*, pages 36-37), or pixyl groups (*Id.*, page 65). A preferred acid labile protecting group is a substituted or unsubstituted trityl, for example, 4,4'-dimethoxytrityl (hereinafter "DMT"). Trityl groups are preferably, substituted by electron donating substituents such as alkoxy groups.

When the H-phosphonate is a protected deoxyribonucleoside, ribonucleoside, oligodeoxyribonucleotide, or oligoribonucleotide derivative comprising a 5' H-phosphonate function, the 3' hydroxy function is advantageously protected by a suitable protecting group. Suitable protecting groups include those disclosed above for the protection of the 5' hydroxy functions of 3' H-phosphonates and acyl groups, such as levulinoyl and substituted levulinoyl groups.

When the H-phosphonate is a protected ribonucleoside or a protected oligoribonucleotide, the 2'-hydroxy function is advantageously protected by a suitable protecting group, for example, an acid-labile acetal protecting group, particularly a 1-(aryl)-4-alkoxypiperidin-4-yl group such as 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl (Fpmp) or 1-(2-chlorophenyl)-4-ethoxypiperidin-4-yl (Cpep); and trialkylsilyl groups, often tri(C_{1-4}-alkyl)silyl groups such as a tertiary butyl dimethylsilyl group. Alternatively, the ribonucleoside or oligoribonucleotide may be a 2'-O-alkyl, 2'-O-alkoxyalkyl, or 2'-O-alkenyl derivative, commonly a C_{1-4} alkyl, C_{1-4} alkoxy, C_{1-4} alkyl, or alkenyl derivative, in which case, the 2' position does not need further protection. H-phosphonates of nucleoside and oligonucleotide analogs that may be employed in the process of the present invention include 2'-fluoro, 2'-amino, 2'-C-alkyl, and 2'-C-alkenyl substituted nucleoside and oligonucleotide derivatives.

Other H-phosphonates that may be employed in the methods of the present invention are derived from other polyfunctional alcohols, especially alkyl alcohols, and preferably, diols or triols. Examples of alkyl diols include ethane-1,2-diol, and low molecular weight poly(ethylene glycols), such as those having a molecular weight of up to 400. Examples of alkyl triols include glycerol and butane triols. Commonly, only a single H-phosphonate function will be present, the remaining hydroxy groups being protected by suitable protecting groups, such as those disclosed hereinabove for protection at the 5' or 2' positions of ribonucleosides.

H-phosphonate multimers, for example, dimers or trimers are another example of H-phosphonates that can be used in the invention as described herein. The multimers may contain an H-phosphonate backbone, or may be derivatized to form, for example, a

phosphodiester backbone, or a phosphorothioate diester backbone, using methods described herein.

The H-phosphonate is coupled with a substrate comprising a free hydroxy group, often a nucleoside or an oligonucleotide, which may be protected or unprotected, preferably comprising a free 5' or 3' hydroxy function, and more preferably comprising a free 5' hydroxy function. When the substrate comprising a free hydroxy group is a protected nucleoside or a protected oligonucleotide, preferred nucleosides are 2'-deoxyribonucleosides and ribonucleosides, for example, 2'-O-(alkyl, alkyloxyl, or alkenyl)-ribonucleosides and preferred oligonucleotides are oligo(2'-deoxyribonucleotides) and oligoribonucleotides, for example, 2'-O-(alkyl, alkyloxyl, or alkenyl)-oligoribonucleotides.

For synthesis of the H-phosphonate diester in solution, when the substrate comprising a free hydroxy group is a deoxyribonucleoside, ribonucleoside, oligodeoxyribonucleotide, or oligoribonucleotide derivative comprising a free 5'-hydroxy group, the 3'-hydroxy function may be protected by a suitable protecting group. Examples of such protecting groups include acyl groups, commonly comprising up to 16 carbon atoms, such as those derived from gamma keto acids, such as levulinoyl groups and substituted levulinoyl groups. Substituted levulinoyl groups include, for example, 5-halo-levulinoyl, such as 5,5,5-trifluorolevulinoyl and benzoylpropionyl groups. Other such protecting groups include fatty alkanoyl groups, for example, linear or branched C_{6-16} alkanoyl groups, such as lauroyl groups; benzoyl and substituted benzoyl groups, such as alkyl, commonly C_{1-4} alkyl-, and halo, commonly chloro or fluoro, substituted benzoyl groups; and silyl ethers, such as alkyl, commonly C_{1-4} alkyl, and aryl, commonly phenyl, silyl ethers, particularly tertiary butyl dimethyl silyl and tertiary butyl diphenyl silyl groups.

For synthesis of an oligonucleotide on a solid support, when the substrate comprising a free hydroxy group is a deoxyribonucleoside, ribonucleoside, oligodeoxyribonucleotide, or oligoribonucleotide derivative comprising a free 5'-hydroxy group, the substrate comprising a free hydroxy group is preferably bonded to the solid support via the 3'-hydroxy function. Alternatively, when the substrate comprising a free hydroxy group is a deoxyribonucleoside, ribonucleoside, oligodeoxyribonucleotide, or oligoribonucleotide derivative comprising a free 3'-hydroxy group, the substrate comprising a free hydroxy group is preferably bonded to the solid support via the 5'-hydroxy function.

When the substrate comprising a free hydroxy group is a protected deoxyribonucleoside, ribonucleoside, oligodeoxyribonucleotide, or oligoribonucleotide comprising a free 3'-hydroxy group, the 5'-hydroxy function is advantageously protected by a suitable protecting group. Suitable protecting groups are those disclosed above for the protection of the 5' hydroxy group of deoxyribonucleosides, ribonucleosides, oligodeoxyribonucleotides, and oligoribonucleotide 3' H-phosphonates.

When the substrate comprising a free hydroxy group is a ribonucleoside or an oligoribonucleotide, the 2'-hydroxy function is advantageously protected by a suitable protecting group, such as an acetal, particularly 1-(2-fluorophenyl)-4-methoxypiperidine-4-yl (Fpmp); and trialkylsilyl groups, often tri(C_{1-4}-alkyl) silyl groups such as a tertiary butyl dimethyl silyl group. Alternatively, the ribonucleoside or oligoribonucleotide may be a 2'-O-alkyl, 2'-O-alkoxyalkyl, or 2'-O-alkenyl derivative, commonly a C_{1-4} alkyl, C_{1-4} alkoxy, or C_{1-4} alkyl or alkenyl derivative, in which case, the 2' position does not need further protection.

Other substrates comprising a free hydroxy group that may be employed in the process according to the present invention are non-saccharide polyols, especially alkyl polyols, and preferably, diols or triols. Examples of alkyl diols include ethane-1,2-diol, and low molecular weight poly(ethylene glycols), such as those having a molecular weight of up to 400. Examples of alkyl triols include glycerol and butane triols. Commonly, only a single free hydroxy group will be present, the remaining hydroxy groups being protected by suitable protecting groups, such as those disclosed hereinabove for the protection at the 5' or 2' positions of ribonucleosides. However, more than one free hydroxy group may be present if it is desired to perform identical couplings on more than one hydroxy group.

In addition to the presence of hydroxy protecting groups, bases present in nucleosides/nucleotides employed in the present invention are also preferably protected where necessary by suitable protecting groups. Protecting groups employed are those known in the art for protecting such bases. For example, A and/or C can be protected by benzoyl, including substituted benzoyl, for example, alkyl- or alkoxy-, often C_{1-4} alkyl- or C_{1-4} alkoxy-; benzoyl; pivaloyl; and amidine, particularly dialkylaminomethylene, preferably, di(C_{1-4}-alkyl) aminomethylene, such as dimethyl or dibutyl aminomethylene. O6 of G may be protected by a phenyl group, including a substituted phenyl, for example, 2,5-dichlorophenyl and diphenyl carbamoyl, and also by an isobutyryl group. T and U generally do not require protection, but in certain embodiments may advantageously be protected, for example, at O4 by a phenyl group, including a substituted phenyl, for example, 2,4-dimethylphenyl or at N3 by a pivaloyloxymethyl, benzoyl, alkyl, or alkoxy substituted benzoyl, such as C_{1-4} alkyl- or C_{1-4} alkoxybenzoyl.

When the substrate comprising a free hydroxy group and/or an H-phosphonate is a protected nucleoside or oligonucleotide having protected hydroxy groups, one of these protecting groups may be removed after carrying out the coupling reaction described above. Commonly, the protecting group removed is that on the 3'-hydroxy function. After the protecting group has been removed, the oligonucleotide thus formed may be converted into an H-phosphonate and may then proceed through one or more additional coupling reactions and one or more derivatizations, as described herein. The method may then proceed with steps to remove the protecting groups from the internucleotide linkages, the 3' and the 5'-hydroxy groups, and from the bases, if so desired. Similar

methodology may be applied to coupling 5' H-phosphonates, wherein the protecting group removed is that on the 5' hydroxy function.

Protecting groups can be removed using methods known in the art for the particular protecting group and function. For example, transient protecting groups, commonly employed on 3'-hydroxy groups, particularly gamma keto acids such as levulinoyl-type protecting groups, can be removed by treatment with hydrazine, for example, buffered hydrazine, such as the treatment with hydrazine under very mild conditions disclosed by van Boom. J.H.; Burgers, P.M.J. *Tetrahedron Lett.*, 1976, 4875-4878. The resulting partially-protected oligonucleotides with free 3'-hydroxy functions may then be converted into the corresponding H-phosphonates, which are intermediates that can be employed for the block synthesis of oligonucleotides and their phosphorothioate analogues.

Other base protecting groups, for example, benzoyl, pivaloyl, and amidine groups can be removed by treatment with concentrated aqueous ammonia.

Trityl groups present can be removed by treatment with acid. With regard to the overall unblocking strategy in oligodeoxyribonucleotide synthesis, another important consideration of the present invention is that the removal of trityl, often a 5'-terminal DMTr protecting group ('detritylation') should proceed without concomitant depurination, especially of any 6-*N*-acyl-2'-deoxyadenosine residues. According to an embodiment of the invention, such depurination, which perhaps is difficult completely to avoid in solid phase synthesis, can be totally suppressed by effecting 'detritylation' with a dilute solution of hydrogen chloride at low temperature, particularly about 0.45 M hydrogen chloride in dioxane - dichloromethane (1:8 v/v) solution at -50°C. Under these reaction conditions, 'detritylation' can be completed rapidly, and in certain cases after 5 minutes or less. For example, when 6-*N*-benzoyl-5'-*O*-(4,4'-dimethoxytrityl)-2'-deoxyadenosine was treated with hydrogen chloride in dioxane - dichloromethane under such conditions, 'detritylation' was complete after 2 minutes, but no depurination was detected even after 4 hours.

Silyl protecting groups may be removed by fluoride treatment, for example, with a solution of a tetraalkyl ammonium fluoride salt such as tetrabutyl ammonium fluoride.

Fpmp protecting groups may be removed by acidic hydrolysis under mild conditions.

As described above, the coupling of the H-phosphonate to a substrate comprising a free hydroxy group, for example, a nucleoside or a nascent oligonucleotide, occurs in the presence of a compound represented by Structural Formula I. In a preferred embodiment, in the compound represented by Structural Formula I, Z is -Cl; X^7 is =O; R^1 is =O; R^2 is -O-; R^3 is H; and n is 1, as shown in FIG. 1. The coupling reaction is conveniently carried out at a temperature in the range of approximately 15°C to about 30°C, and this range has been used for solution and solid phase.

The newly formed H-phosphonate diesters or oligonucleotides generated as described herein can be derivatized to form, for example, phosphodiesters or phosphorothioate triesters. Such derivatizations are carried out *in situ* or once the oligonucleotide is completely synthesized. As used herein, by "*in situ*" is meant that the product of the coupling reaction is derivatized without separation and purification of the intermediate produced by the coupling reaction. For example, protected nucleosides or oligonucleotides with a 3'-terminal H-phosphonate function and protected nucleosides or oligonucleotides with a 5'-terminal hydroxy function are coupled in the presence of a compound represented by Structural Formula I to form an oligonucleoside or oligonucleotide H-phosphonate intermediate, where the intermediates undergo sulfur-transfer in the presence of a suitable sulfur-transfer agent.

In situ derivatization of the H-phosphonate occurs through the use of a sulfur transfer agent. The nature of the sulfur-transfer agent will depend on whether an oligonucleotide having phosphodiester linkages, a phosphorothioate analog or a mixed oligonucleotide/oligonucleotide phosphorothioate (chimeric oligonucleotide) is desired.

Sulfur transfer agents employed in the process of the present invention often have the general chemical formula:

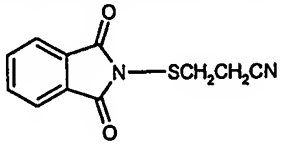
L——S——A

wherein L represents a leaving group, and A represents an aryl group, a methyl group, a substituted alkyl group or an alkenyl group. Commonly the leaving group is selected so as to comprise a nitrogen-sulfur bond. Examples of suitable leaving groups include morpholines such as morpholine-3,5-dione; imides such as phthalimides, succinimides and maleimides; indazoles, particularly indazoles with electron-withdrawing substituents such as 4-nitroindazoles; and triazoles.

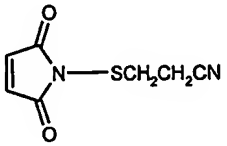
Where a phosphorothioate diester linkage is desired in the final product, the moiety A represents a methyl, substituted alkyl, or alkenyl group. Examples of suitable substituted alkyl groups include substituted methyl groups, particularly benzyl and substituted benzyl groups, such as alkyl-, commonly C_{1-4}alkyl- and halo-, commonly chloro-, substituted benzyl groups, and substituted ethyl groups, especially ethyl groups substituted at the 2-position with an electron-withdrawing substituent such as 2-(4-nitrophenyl)ethyl and 2-cyanoethyl groups. Examples of suitable alkenyl groups are allyl and crotyl. Examples of a suitable class of phosphorothioate-directing sulfur-transfer agents are, for example, (2-cyanoethyl)sulfanyl derivatives such as 4-[(2-cyanoethyl)-sulfanyl]morpholine-3,5-dione, or a corresponding reagent such as 3-(phthalimidosulfanyl)propanonitrile.

For example, a phosphorothioate triester can be formed by reacting a nucleoside H-phosphonate with a substrate comprising a free hydroxy group in the presence of a

coupling reagent represented by Structural Formula I, in the presence of a compound represented by one of Structural Formulae II, III, or IV:



II



III

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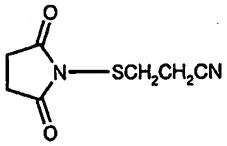


IV

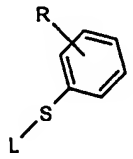
When deprotecting the desired product once it has been produced, protecting groups on the phosphorus backbone that produce phosphorothioate triester linkages are commonly removed first. For example, a cyanoethyl group can be removed by treatment with a strongly basic amine such as DABCO, 1,5-diazabicylo[4.3.0]non-5-ene (DBN), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or triethylamine.

Where a standard phosphodiester linkage is desired in the final product, in the sulfur transfer agent, the moiety A represents an aryl group, such as a phenyl or naphthyl group. Examples of suitable aryl groups include substituted and unsubstituted phenyl groups, particularly halophenyl and alkylphenyl groups, especially 4-halophenyl and 4-alkylphenyl, commonly 4-(C_{1-4} alkyl)phenyl groups, most preferably, 4-chlorophenyl and p-tolyl groups. An example of a suitable class of standard phosphodiester-directing sulfur-transfer agent is an *N*-(arylsulfanyl)phthalimide (succinimide or other imide may also be used).

Once the oligonucleotide is completely synthesized using the above described coupling and *in situ* derivatization methods, phenyl and substituted phenyl groups on the phosphorothioate internucleotide linkages and on the base residues can be removed by oximate treatment, for example, with the conjugate base of an aldoxime, preferably that of *E*-2-nitrobenzaldoxime or pyridine-2-carboxaldoxime (Reese *et al.*, Nucleic Acids Res. 1981) to form a phosphotriester. Kamimura *et al.* in *J. Am. Chem. Soc.*, 1984, 106 4552-4557 and Sekine *et al.* in *Tetrahedron*, 1985, 41, 5279-5288 in an approach to oligonucleotide synthesis by the phosphotriester approach in solution, based on *S*-phenyl phosphorothioate intermediates; and van Boom and his co-workers in an approach to oligonucleotide synthesis, based on *S*-(4-methylphenyl) phosphorothioate intermediates

(Wreesman *et al.*, *Tetrahedron Lett.*, 1985, 26, 933-936) have all demonstrated that unblocking S-phenylphosphorothioates with oximate ions (using the method of Reese *et al.*, 1978; and Reese and Zard, *Nucleic Acids Res.*, 1981, 9, 4611-4626) led to natural phosphodiester internucleotide linkages.

For example, a phosphotriester can be formed by reacting a nucleoside H-phosphonate with a substrate comprising a free hydroxy group in the presence of a coupling reagent represented by Structural Formula I, in the presence of a compound represented by Structural Formula V:



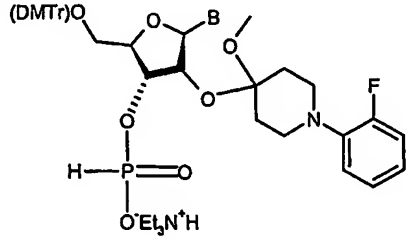
V.

wherein L is a leaving group as described above, and R is -H, an alkyl group, or a halogen.

Once the entire oligonucleotide has been synthesized, it is reacted with a base and an oximate to form a phosphodiester.

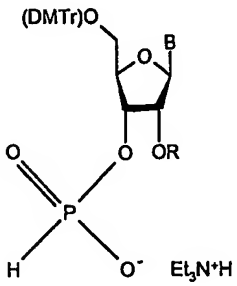
Chimeric oligonucleotides containing both phosphodiester and phosphorothioate diester linkages can also be generated using the above reagents. For example, a chimeric oligonucleotide can be formed by reacting a nucleoside H-phosphonate with a substrate comprising a free hydroxy group in the presence of a coupling reagent represented by Structural Formula I, in the presence of a compound represented by Structural Formula V. After one or more coupling steps, the oligonucleotide is contacted with any of the compounds represented by Structural Formulae II, III, or IV. The oligonucleotide is then reacted with a base and an oximate to form a chimeric oligonucleotide.

As stated previously, the method of the invention can be used in the synthesis of RNA, 2'-O-alkyl-RNA, 2'-O-alkoxyalkyl-RNA and 2'-O-alkenyl-RNA sequences. 2'-O-(Fpmp)-5'-O-(4,4-dimethoxytrityl)-ribonucleoside 3'-H-phosphonates, an example of which is shown in Structural Formula VI:



(VI)

and 2'-O-(alkyl, alkoxyalkyl or alkenyl)-5'-O-(4,4-dimethoxytrityl)-ribonucleoside 3'-H-phosphonates, an example of which is shown in Structural Formula VII;



a: R = Me

b: R = CH_2=$CHCH_2$

c: R = $MeOCH_2CH_2$

(VII)

may be prepared, for example, from the corresponding nucleoside building blocks, ammonium *p*-cresyl H-phosphonate and a compound represented by Structural Formula I.

For solution phase synthesis, a suitable temperature for carrying out the coupling reaction and *in situ* derivatization step is in the range of about -55°C to about 35°C. Preferably, the temperature is in the rage of about 0°C to about 30°C. Most preferably, room temperature (commonly in the range of from about 10°C to about 25°C, for example, from about 20°C to about 25°C) is used.

For solid support synthesis, a suitable temperature for carrying out the coupling reaction and *in situ* derivatization step is in the range of about -55°C to about 40°C. Preferably, the temperature is in the rage of about 0°C to about 30°C. Most preferably, room temperature (commonly in the range of from about 10°C to about 25°C, for example, from about 20°C to about 25°C) is used.

When solution phase synthesis is employed, organic solvents, which can be employed in the process of the present invention include haloalkanes, particularly dichloromethane, esters, particularly alkyl esters such as ethyl acetate, and methyl or ethyl propionate, and basic, nucleophilic solvents such as pyridine. Preferred solvents for the coupling and *in situ* sulfur transfer steps are pyridine, dichloromethane, and mixtures thereof. Other preferred solvents include dimethylformamide, N–methylpyrollidinone, and mixtures thereof.

Solid supports that are employed in the methods according to the present invention are substantially insoluble in the solvent employed, and include those supports well known in the art for the solid phase synthesis of oligonucleotides. Examples include silica, controlled pore glass, polystyrene, copolymers comprising polystyrene such as

polystyrene-poly(ethylene glycol) copolymers; and polymers such as polyvinylacetate. Additionally, poly(acrylamide) supports, especially microporous or soft gel supports, such as those more commonly employed for the solid phase synthesis of peptides may be employed if desired. Preferred poly(acrylamide) supports are amine-functionalized supports, especially those derived from supports prepared by copolymerisation of acryloyl-sarcosine methyl ester, N,N-dimethylacrylamide and bis-acryloylethylenediamine, such as the commercially available (Polymer Laboratories) support sold under the catalogue name PL-DMA. The procedure for preparation of the supports has been described by Atherton and Sheppard in Solid Phase Synthesis: A Practical Approach, Publ., IRL Press at Oxford University Press (1984). The functional group on such supports is a methyl ester and this is initially converted to a primary amine functionality by reaction with an alkyl diamine, such as ethylene diamine.

The substrate is commonly bound to the solid support via a cleavable linker. Examples of linkers that may be employed include those well known in the art for the solid phase synthesis of oligonucleotides, such as urethane, oxalyl, succinyl, and amino-derived linkers.

In many embodiments when the substrate is bound to a poly(acrylamide) support via a cleavable linker and comprises a nucleoside, the substrate is attached to the support by a process comprising either:

a) reacting a 5'-protected nuceloside having a free 3'-hydroxy group with a linker, preferably succinic anhydride, to form a linker-derivatized nucleoside; and

b) reacting the linker-derivatized nucleoside with an amine-functionalized poly(acrylamide) support in the presence of a coupling agent used for amide bond formation and optionally a catalyst, such as a base, for example, diisopropylethylamine (DIPEA) or N-methylmorpholine (NMM), or hydroxybenzotriazole; or

c) reacting an amine-functionalized poly(acrylamide) support with a linker, preferably succinic anhydride, to form a linker-derivatized support; and

d) reacting the linker-derivatized support with a 5'-protected nucleoside having a free 3'-hydroxy group in the presence of a coupling agent used for amide bond formation and optionally a catalyst, such as a base, for example, DIPEA or NMM, or hydroxybenzotriazole;

then in either case, removing the 5'-protecting group, which is preferably a trityl or substituted trityl group. However, it will be recognized that it may be desirable to retain the 5'-protecting group, in which case its removal may be omitted. In this case, the 5'-protecting group can be removed when desired prior to use of the supported substrate in the process for the synthesis of phosphorothioate triesters according to the present invention.

Coupling agents used for amide bond formation that can be employed in the process for attaching the substrate to an amine-functionalized poly(acrylamide) support include those known in the art of peptide synthesis, see, for example, those coupling reagents disclosed by Wellings, D.A.; Atherton, E.; in Methods in Enzymology, Publ., Academic Press, New York (1997) incorporated herein by reference, such as those comprising carbodiimides, especially dialkyl carbodiimides such as N,N'-diisopropylcarbodiimide (DIC), and reagents that form active esters, particularly benzotriazole active esters *in situ*, such as 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) or benzotriazole-1-yloxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP).

An organic solvent such as N,N-dimethylformamide (DMF) or N-methylpyrrolidinone (NMP) is suitably employed for attaching the substrate to an amine-functionalized poly(acrylamide) support.

The process for the synthesis of phosphodiesters or phosphorothioate triesters according to the present invention can be carried out by stirring a slurry of the substrate bonded to the solid in a solution of the H-phosphonate and coupling agent or sulfur transfer agent. Alternatively, the solid support can be packed into a column, and solutions of H-phosphonate and coupling agent, followed by sulfur transfer agent can be passed sequentially through the column.

The mole ratio of H-phosphonate to substrate comprising a free hydroxy group in the process of the present invention is often selected to be in the range of from about 0.9:1 to 3:1, commonly from about 1:1 to about 2:1, and preferably, from about 1.1:1 to about 1.5:1, such as about 1.2:1. However, where couplings on more than one free hydroxyl are taking place at the same time, the mole ratios will be increased proportionately. The mole ratio of a compound represented by Structural Formula I to substrate comprising a free hydroxy group is often selected to be in the range of from about 1:1 to about 10:1, commonly from about 1.5:1 to about 5:1 and preferably, from about 1.5:1 to about 3:1. The mole ratio of oxidizing agent or sulfur transfer agent to substrate comprising a free hydroxy group is often selected to be in the range of from about 1:1 to about 10:1, commonly from about 2:1 to about 5:1 and preferably, from about 2:1 to about 3:1.

The above same protocols are used as in the synthesis of DNA and DNA phosphodiester or phosphorothioate sequences *in situ*, and are also described, for example, in WO 99/09041, the teachings of which are incorporated by reference in their entirety herein. Following the standard unblocking procedure, the Fpmp protecting groups are removed under mild conditions of acidic hydrolysis that lead to no detectable cleavage or migration of the internucleotide linkages (Capaldi and Reese,. *Nucleic Acids Res.* 1994, 22, 2209-2216). For chemotherapeutically useful ribozyme sequences, relatively large scale RNA synthesis in solution is a matter of considerable practical importance. The

incorporation of 2'-O-alkyl, 2'-O-substituted alkyl and 2'-O-alkenyl, especially 2'-O-methyl, 2'-O-allyl and 2'-O-(2-methoxyethyl)]-ribonucleosides (Sproat, 'Methods in Molecular Biology, Vol. 20. Protocols for Oligonucleotides and Analogs', Agrawal, Ed., Humana Press, Totowa, 1993) into oligonucleotides is currently a matter of much importance, as these modifications confer both resistance to nuclease digestion and good hybridization properties on the resulting oligomers.

H-phosphonates formed using the coupling agent of the present invention can also be derivatized to phosphodiesters or phosphorothioate triesters through oxidation or oxidative sulfurization techniques once the H-phosphonate oligonucleotide has been completely synthesized. When a phosphodiester linkage is desired, the oxidation reaction is often carried out by treating the H-phosphonate with an oxidizing agent, such as I_2, in the presence of water and a tertiary amine. Typically, I_2 is added to the reaction mixture so that it is present in about a 0.5 M to about 3.0 M concentration. Water is also added to the reaction mixture so that it is present in about 1% (V/V) to about 10% (V/V). If a tertiary amine is not already present in the reaction mixture, it is also added so that it is present in about 5% to about 20%. Other oxidizing agents can be used in place of I_2, for example, N-chlorosuccinimide, N-bromosuccinimide, salts of periodic acid, or a salt of meta chloroperbenzoic acid. After about 5 minutes to about 20 minutes, the reaction mixture is poured into an aqueous solution of sodium bisulfite to quench the excess iodine, then extracted into an organic solvent to obtain the phosphodiester oligonucleotide.

In another example, derivatizing the H-phosphonate oligonucleotide backbone can be accomplished by contacting the newly formed H-phosphonate diester with an oxidizing agent, a compound represented by R^{12}-X^6-H, and optionally, a base tertiary amine, where R^{12} is a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted aralkyl group; and X^6 is -O-, -S-, or -NR^{10}-, where R^{10} is H, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted aralkyl group; or X^6 is -NR^{10}-, and R^{12} and R^{10} taken together with the nitrogen to which they are attached form a heterocycloalkyl.

Alternatively, the phosphodiester linkages can undergo oxidative sulfurization to form a phosphorothioate triester, using a sulfur transfer reagent, such as *3H*-benzodithiol-3-one 1,1-dioxide (also called "Beaucage reagent"), dibenzoyl tetrasulfide, phenylacetyl disulfide, N,N,N',N'-tetraethylthiuram disulfide, 3-amino-1,2,4-dithiazole-5-thione, or elemental sulfur.

In another example, a phosphorothioate triester is formed by derivatizing the newly formed H-phosphonate diester with elemental sulfur and a tertiary amine.

In the methods of the present invention, it is possible to prepare chimeric oligonucleotides containing both phosphodiester and phosphorothioate diester internucleotide linkages in the same molecule by selection of appropriate sulfur transfer agents, particularly when the process is carried out in a stepwise manner. For example,

one or more reaction cycles are performed, ending with a condensation step to form a nascent H-phosphonate oligonucleotide. The nascent H-phosphonate oligonucleotide is then contacted with an oxidizing agent such as I_2 or CCl_4, and water in a first derivatization step. The oxidizing agent is typically dissolved in a polar organic solvent such as pyridine, acetonitrile, dimethylformamide, an ether (e.g., tetrahydrofuran and dioxane), or combinations thereof. When the oxidizing agent is dissolved in a non-basic solvent a tertiary amine, such as N-methylimidazole, a trialkylamine (e.g., triethylamine, trimethylamine) diisopropylethylamine and the like, must also be present, often in an amount of from about 5% (V/V) to about 40% (V/V) in the derivatization reaction mixture.

The oxidizing agent is present in at least 1 equivalent in relationship to the H-phosphonate groups to be derivatized. However, it can be present in a large excess which is only limited by its solubility in a particular solvent in which the derivatization reaction is preformed. Typically, the oxidizing agent is present in the reaction mixture in about 2 equivalents to about 10 equivalents in relationship to H-phosphonate groups to be derivatized. The H-phosphonate oligonucleotide is contacted with the derivatizing reaction mixture for about 5 minutes to about 1 hour to form a nascent oligonucleotide having a phosphodiester backbone.

The nascent derivatized oligonucleotide is then subjected to one or more additional reaction cycles ending with a condensation step to form an oligonucleotide in which part of the backbone is a phosphodiester backbone and the other part is an H-phosphonate backbone. The oligonucleotide is then contacted with a suitable sulfur transfer agent, or elemental sulfur, for example, those described herein. The second derivatization step is performed in the same manner as the first derivatization step described above, and the result is chimeric oligonucleotide having the desired phosphorothioate diester and phosphodiester linkages. The order in which these two steps occur may be reversed. For example, the oligonucleotide can be derivatized first by sulfurization, followed by oxidation. In addition, the first and second derivatization steps can be repeated one or more times. After the synthesis of the chimeric oligonucleotide is complete, the protecting groups can be removed by standard methods, such as treatment with an ammonium hydroxide solution.

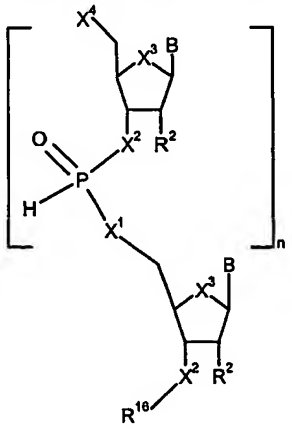
Other methods for derivatizing the oligonucleotide are known to those skilled in the art and are included in the present invention. For further review of methods of derivatizing H-phosphonate oligonucleotides see Froehler, "Oligodeoxynucleotide Synthesis," in *Methods in Molecular Biology*, Vol. 20, *Protocols for Oligonucleotides and Analogs*, p. 63-80, Agrawal, Ed., Humana Press, 1993; Uhlmann and Peyman, Chem. Rev. 90:543, 1990; and Iyer *et al.*, J. Org. Chem. 55:4693, 1990, the entire contents of which are incorporated herein by reference. The formation of the H-phosphonate linkage can be carried out in 10% N-methylimidazole solution, preferably in dry pyridine. Other solvents

such as acetonitrile and methylene chloride can also be used for the coupling and *in situ* sulfur transfer reaction.

When it is desired to obtain a product in which the 5'-end group is protected, the final step of the reaction cycle is a coupling step (or a derivatization step if a phosphodiester or phosphorothioate is desired as the final product). If a 5'-deprotected product is desired, the reaction cycle can end with the deprotection step. Usually, a 5'-protected H-phosphonate or oligonucleotide is the desired product if it is to be purified by reverse phase high performance liquid chromatography (HPLC) or ion-exchange chromatography. If the H-phosphonate or oligonucleotide is to be purified by ion-exchange chromatography or electrophoresis, a 5'-deprotected oligonucleotide is preferred even for ion-exchange chromatography. The 5'-protecting group provides additional separation between failure/shorter sequences from the desired full-length desired oligonucleotide.

Each of the methods described above for coupling, protecting, deprotecting, or derivatizing an H-phosphonate diester, either in solution or on a solid support can be applied to generate oligonucleotides. For example, the present invention features a method for producing an oligonucleotide H-phosphonate, comprising reacting an oligonucleotide comprising a free hydroxy function, with a substituted or unsubstituted alkyl H-phosphonate salt or a substituted or unsubstituted aryl H-phosphonate salt in the presence of a coupling agent represented by Structural Formula I. Preferably, the oligonucleotide comprising a free hydroxy function has a free 3' or 5' hydroxy function. In addition, preferably, the oligonucleotide is a protected oligodeoxyribonucleotide. In another preferred embodiment, the H-phosphate salt is an ammonium salt of a phenyl, alkylphenyl, or halophenyl H-phosphonate.

The coupling methods and coupling reagent, as described above can be used to prepare an oligonucleotide as follows. The first step in the method involves condensing a nucleoside or a nascent oligonucleotide represented by Structural Formula VIII:



VIII

with a monomer by reacting the two reagents in the presence of a coupling reagent represented by Structural Formula I. Preferably, the coupling reagent represented by Structural Formula I is that shown in FIG. 1. In Structural Formula VIII, X^1 for each occurrence is, independently, -O- or -S-. Preferably, X^1 is -O- at every occurrence. X^2 for each occurrence is, independently, -O-, -S-, $-CH_2-$, or $-NR^{11}$. Preferably, X^2 is -O- at every occurrence. X^3 for each occurrence is, independently, -O-, -S-, $-CH_2-$, or $-(CH_2)_2-$. Preferably, X^3 is -O- at every occurrence. In a more preferred embodiment, X^1, X^2, and X^3 are all -O- at every occurrence. X^4 is -OH or -SH. Preferably, X^4 is -OH. R^2 is -H, -F, $-OR^6$, $-NR^7R^8$, or $-SR^9$. R^6 is -H, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, a substituted or unsubstituted aralkyl group, or a hydroxy protecting group. R^7 and R^8 are each, independently, -H, a substituted or substituted aliphatic group, or an amine protecting group. R^9 is -H, a substituted or unsubstituted aliphatic group, or a thio protecting group. R^{11} is -H, an alkyl group, an aryl group, or an aralkyl group. R^{16} is a hydroxy protecting group, a thio protecting group, an amino protecting group, a solid support, or a cleavable linker attached to a solid support, such as a group of the formula $-Y^2-L-Y^2-R^{15}$. Amine, hydroxy, and thiol protecting groups are known to those skilled in the art. For examples of amine protecting groups see Greene *et al.* (*Protective Groups in Organic Synthesis* (1991), John Wiley & Sons, Inc., pages 309-405), the teachings of which are incorporated herein by reference in their entirety. Preferably, amines are protected as amides or carbamates. For examples of alcohol protecting groups see *Id.*, pages 10-142, the teachings of which are incorporated herein by reference in their entirety. A preferred hydroxy protecting group is *t*-butyldimethylsilyl group. For examples of thiol protecting groups see *Id.*, pages 277-308, the teachings of which are incorporated herein by reference in their entirety.

In addition, in Structural Formula VIII, Y^2 for each occurrence is, independently, a single bond, -C(O)-, $-C(O)NR^{17}-$, -C(O)O-, $-NR^{17}-$, or -O-. L is a linker that is preferably, a substituted or unsubstituted aliphatic group or a substituted or unsubstituted aromatic group. More preferably, L is an ethylene group. R^{17} is -H, a substituted or unsubstituted aliphatic group or a substituted or unsubstituted aromatic group. R^{15} is a solid support, such as controlled-pore glass or polystyrene. B for each occurrence is, independently, H or a protected or unprotected nucleoside base. n is zero or a positive integer.

Aliphatic groups, as used herein, include straight chained or branched C_1-C_{18} hydrocarbons that are completely saturated or that contain one or more unconjugated double bonds, or cyclic C_3-C_{18} hydrocarbons that are completely saturated or that contain one or more unconjugated double bonds. Alkyl groups are straight chained or branched C_1-C_8 hydrocarbons or C_3-C_8 cyclic hydrocarbons that are completely saturated. Aliphatic groups are preferably alkyl groups.

Aryl groups include carbocyclic aromatic ring systems (e.g., phenyl) and carbocyclic aromatic ring systems fused to one or more carbocyclic aromatics (e.g.,

naphthyl and anthracenyl) or an aromatic ring system fused to one or more non-aromatic rings (e.g., 1,2,3,4-tetrahydronaphthyl).

Heteroaryl groups, as used herein, include aromatic ring systems that have one or more heteroatoms selected from sulfur, nitrogen, or oxygen in the aromatic ring (e.g., thienyl, pyridyl, pyrazole, isoxazolyl, thiadiazolyl, oxadiazolyl, indazolyl, furans, pyrroles, imidazoles, pyrazoles, triazoles, pyrimidines, pyrazines, thiazoles, isoxazoles, isothiazoles, tetrazoles, oxadiazoles, benzo(b)thienyl, benzimidazole, indole, tetrahydroindole, azaindole, indazole, quinoline, imidazopyridine, purine, pyrrolo[2,3-d]pyrimidine, and pyrazolo[3,4-d]pyrimidine). Preferably, heteroaryl groups are five or six membered ring systems having from one to four heteroatoms.

Azaheteroaryl compounds, as used herein, include heteroaryl groups which have one or more nitrogen atoms in the aromatic ring. Preferably, azaheteroaryl compounds have five or six membered aromatic rings with from one to three nitrogens in the aromatic ring. Preferred azaheteroaryl compounds are organic bases. Examples of azaheteroaryl compounds that are organic bases include pyrimidine, 1-alkylpyrazole, pyrazine, N-alkylpurine, N-alkylpyrrole, pyridine, N-alkylimidazole, quinoline, isoquinoline, quinoxaline, quinazoline, N-alkylindole, N-alkylbenzimidazole, triazine, thiazole, N-alkylindole, and 1-alkyl-7-azaindole.

An aralkyl group, as used herein, is an aromatic substituent that is linked to a moiety by an alkyl group.

A heterocycloalkyl group, as used herein, is a non-aromatic ring system that preferably has five to six atoms and includes at least one heteroatom selected from nitrogen, oxygen, and sulfur.

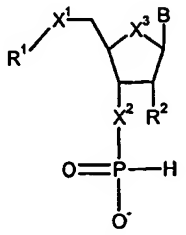
A heterocycloalkyl group, as used herein, is a non-aromatic ring system that preferably has five to six atoms and includes at least one heteroatom selected from nitrogen, oxygen, and sulfur. Examples of heterocycloalkyl groups include morpholinyl, piperidinyl, piperazinyl, thiomorpholinyl, pyrrolidinyl, thiazolidinyl, tetrahydrothienyl, azetidinyl, tetrahydrofuryl, dioxanyl, and dioxepanyl.

Azaheterocycloalkyl groups, as used herein, are heterocycloalkyl groups that have at least one nitrogen atom in the non-aromatic ring system. Examples of azaheterocycloalkyl groups include morpholines, piperidines, and piperazines.

Heterocyclic groups, as used herein, include heteroaryl groups and heterocycloalkyl groups.

Suitable substituents for aliphatic groups, aryl groups, aralkyl groups, heteroaryl groups, azaheteroaryl groups and heterocycloalkyl groups include aryl groups, halogenated aryl groups, alkyl groups, halogenated alkyl (e.g., trifluoromethyl and trichloromethyl), aliphatic ethers, aromatic ethers, benzyl, substituted benzyl, halogens, cyano, nitro, -S-(aliphatic or substituted aliphatic group), and -S-(aromatic or substituted aromatic).

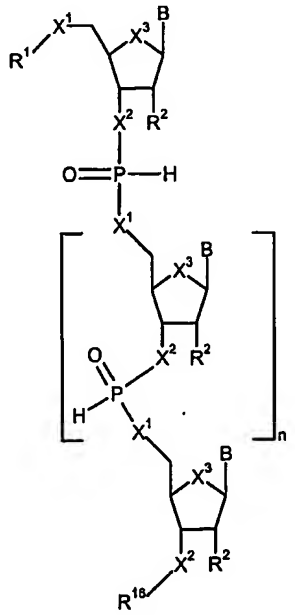
The monomer to which the nucleoside or nascent oligonucleotide is coupled can be represented by Structural Formula IX:



IX.

In Structural Formula IX, X^1, X^2, X^3, R^2, and B are as defined above. R^1 is an alcohol protecting group or a thio protecting group. Preferably, R^1 is an acid labile protecting group.

The nascent oligonucleotide is contacted with the monomer and a compound represented by Structural Formula I and forms a nascent H-phosphonate (n+1) oligonucleotide represented by Structural Formula X:

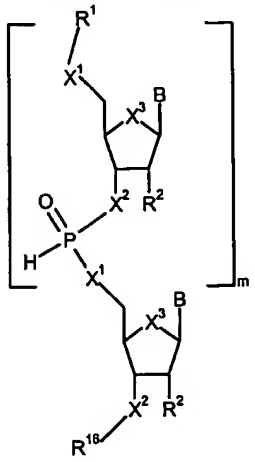


X.

In Structural Formula X, X^1, X^2, X^3, R^1, R^2, R^{16}, B, and n are as defined above. The nascent H-phosphonate (n+1) oligonucleotide can be deprotected by removing the protecting group represented by R^1. If R^1 is an acid labile protecting group, the nascent H-phosphonate (n+1) oligonucleotide is treated with an acid to remove R^1. If R^1 is a

trialkylsilyl group, such as *t*-butyldimethylsilyl group or a triisopropylsilyl group, the nascent H-phosphonate (n+1) oligonucleotide can be treated with fluoride ions to remove R^1. Typically, *t*-butyldimethylsilyl and a triisopropylsilyl are removed by treatment with a solution of tetrabutylammonium fluoride in THF. Methods for removing *t*-butyldimethylsilyl can be found in Greene *et al.* (*Protective Groups in Organic Synthesis* (1991), John Wiley & Sons, Inc., pages 77-83), the teachings of which are incorporated herein by reference in their entirety.

The condensation and deprotection reaction steps, referred to herein as the reaction cycle, can be repeated one or more times to form an H-phosphonate oligonucleotide of the desired length that can be represented by Structural Formula XI:

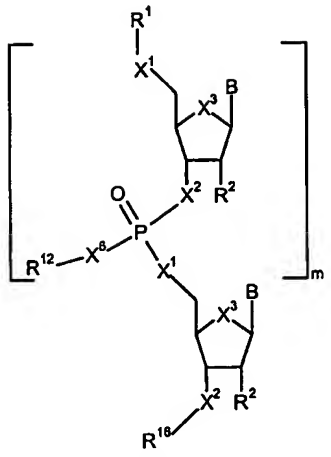


XI.

In Structural Formula XI, X^1, X^2, X^3, R^1, R^2, R^{16}, and B are as defined above; and m is a positive integer.

The H-phosphonate oligonucleotide represented by Structural Formula XI can then be treated with a sulfur transfer agent, as defined above, to form a phosphodiester or a phosphorothioate diester, using methods described above.

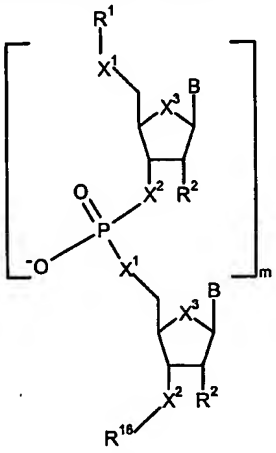
For example, once the oligonucleotide has been completely synthesized, it can be contacted with an oxidizing agent, a compound represented by R^{12}-X^6-H, as defined above, and optionally, a base tertiary amine, thereby forming an oligonucleotide represented by the Structural Formula XII:



XII.

thereby forming a phosphodiester oligonucleotide. In Structural Formula XII, X^1, X^2, X^3, X^6, R^1, R^2, R^{12}, R^{16}, and B are as defined above; and m is a positive integer.

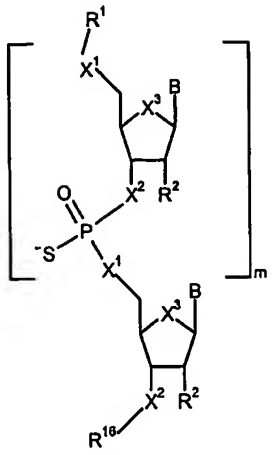
In another example, once the oligonucleotide is completely synthesized, the oligonucleotide backbone is derivatized by contacting the H-phosphonate oligonucleotide with an oxidizing agent, a tertiary amine, and water, thereby forming a phosphodiester oligonucleotide represented by Structural Formula XIII:



XIII.

In Structural Formula XIII, X^1, X^2, X^3, R^1, R^2, R^{16}, and B are as defined above; and m is a positive integer.

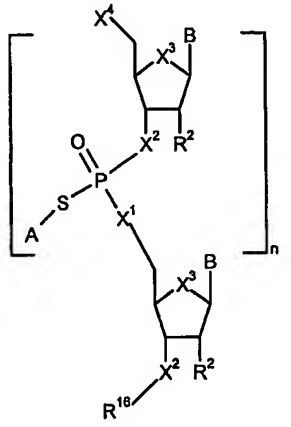
Alternatively, once the H-phosphonate oligonucleotide is completely synthesized, it can be derivatized to form a phosphorothioate. For example, the H-phosphonate oligonucleotide can be contacted with elemental sulfur and a tertiary amine, whereby the oligonucleotide formed is a phosphorothioate represented by Structural Formula XIV:



XIV.

In Structural Formula XIV, X^1, X^2, X^3, R^1, R^2, R^{16}, and B are as defined above; and m is a positive integer.

A second method for forming an oligonucleotide can be carried out as follows. The oligonucleotide is formed by condensing a nucleoside or a nascent oligonucleotide represented by Structural Formula XV:



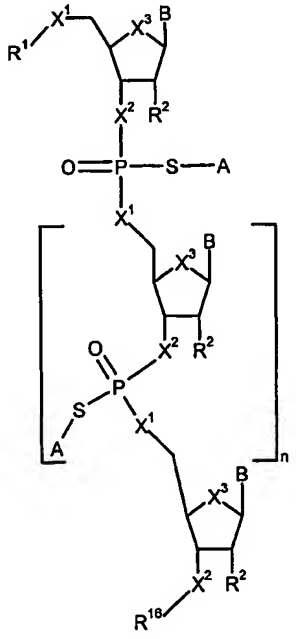
XV.

with a salt of a monomer by reacting the two reagents in the presence of a compound represented by Structural Formula I and a sulfur transfer agent. In Structural Formula XV, X^1, X^2, X^3, X^4, R^1, R^2, R^{16}, and B are as defined above; n is zero or a positive integer; and A is a substituted or unsubstituted aryl group, a substituted or unsubstituted alkyl group, or a substituted or unsubstituted alkenyl group.

The salt of a monomer to which the nucleoside or nascent oligonucleotide is coupled is represented by Structural Formula IX. The sulfur transfer agent used in the reaction is represented by the following general chemical formula:

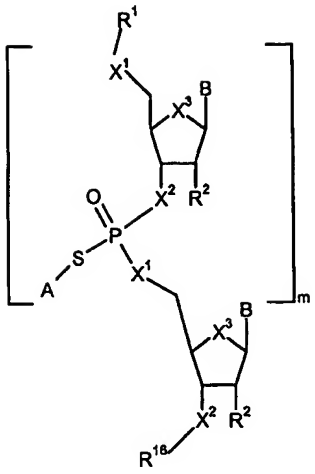
L——S——A

where L represents a leaving group as defined above, and A represents a substituted or unsubstituted aryl group, a substituted or unsubstituted alkyl group, or a substituted or unsubstituted alkenyl group. The nascent H-phosphonate (n+1) oligonucleotide formed is represented by Structural Formula XVI:



XVI.

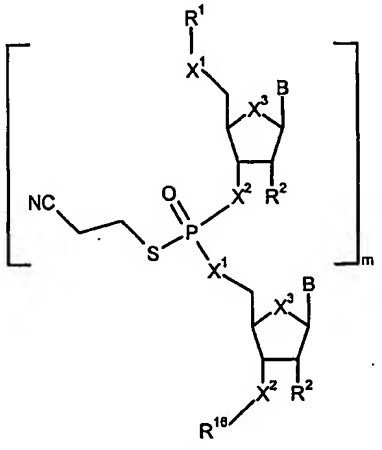
The nascent H-phosphonate (n+1) oligonucleotide can be deprotected as described above, to remove R^1. If desired, the coupling step and deprotection step can be repeated one or more times to form a phosphorothioate oligonucleotide represented by Structural Formula XVII:



XVII.

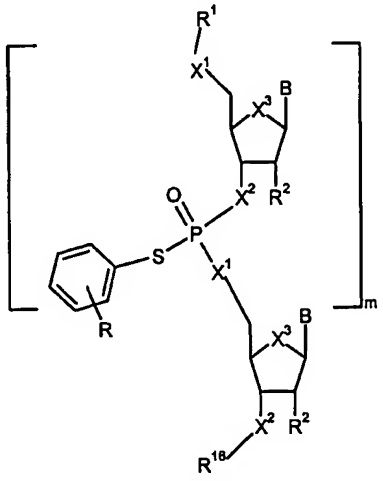
In Structural Formula XVII, X^1, X^2, X^3, R^1, R^2, R^{16}, A, and B are as defined above; and m is a positive integer.

In either of the above methods of generating an oligonucleotide, the leaving group of the sulfur transfer agent is commonly selected so as to comprise a nitrogen-sulfur bond. Examples of suitable leaving groups include morpholines such as morpholine-3,5-dione; imides such as phthalimides, succinimides and maleimides; indazoles, particularly indazoles with electron-withdrawing substituents such as 4-nitroindazoles; and triazoles. In one example of the formation of a phosphorothioate oligonucleotide according to the above methods, the newly formed H-phosphonate oligonucleotide is contacted with a compound having one of Structural Formulae II, III, or IV, thereby forming an oligonucleotide represented by Structural Formula XVIII:



XVIII.

The above described second method can also be used to synthesize a phosphodiester olignucleotide. In this particular method, the sulfur transfer reagent is a compound represented by Structural Formula V, which is used to form a phosphorothioate triester represented by Structural Formula XIX:



XIX.

Once the oligonucleotide is completely synthesized the backbone of the newly formed oligonucleotide is deprotected by contacting the oligonucleotide with an oximate and a base, thereby forming a phosphodiester oligonucleotide.

Alternatively, the above described second method of oligonucleotide synthesis can be use to generate a chimeric oligonucleotide containing both phosphodieser linkages and phosphorothioate diester linkages. This method is carried out as described above, where the H-phosphonate oligonucleotide is contacted with a compound represented by Structural Formula V, and after one or more condensation steps is contacted with one of the compounds represented by Structural Formulae II, II, or IV after one or more different coupling steps, forming a phosphorothioate triester. The phosphorothioate triester backbone of the oligonucleotide is deprotected by contacting the phosphorothioate triester with an oximate and a base, thereby forming a chimeric oligonucleotide.

In any of the above oligonucleotide synthesis methods, the coupling step (or derivatization step, if derivatization of the oligonucleotide is desired) can be the final step, whereby the oligonucleotide generated is a protected oligonucleotide. Alternatively, the deprotection step can be the last step, and the oligonucleotide generated is a deprotected oligonucleotide.

As described above, synthesis of the oligonucleotide can be done in solution or on a solid support. When an H-phosphonate diester or oligonucleotide is formed in solution, R^{16} of each of Structural Formulae VIII and XV is an alcohol, amine, or thiol protecting group. Solution synthesis of the desired oligonucleotide can occur, for example, when the

H-phosphonate and the substrate comprising a free hydroxy group are pre-mixed in solution, and a compound represented by Structural Formula I is then added to this mixture. Alternatively, the H-phosphonate and a compound represented by Structural Formula I can be pre-mixed, often in solution and then added to a solution of the substrate comprising a free hydroxy group, or the substrate comprising a free hydroxy group and a compound represented by Structural Formula I may be mixed, commonly in solution, and then added to a solution of the H-phosphonate. In certain embodiments, the H-phosphonate, optionally in the form of a solution, can be added to a solution comprising a mixture of the substrate comprising a free hydroxy group and a compound represented by Structural Formula I. Reagent additions commonly take place continuously or incrementally over an addition period.

In any of the above described coupling, protecting, deprotecting, and derivatization methods, preferably, the nucleoside or oligonucleotide and/or the monomer is protected. In addition, preferably, in each of Structural Formulae VIII and XV, each R^2 is -OH or -OR^6 and the oligonucleotide prepared is a ribonucleotide. In another preferred embodiment, in each of Structural Formulae VIII and XV, each R^2 is -H and the oligonucleotide prepared is a deoxyribonucleotide.

The present invention is further illustrated without limitation by the following examples.

EXAMPLE 1

A compound represented by Structural Formula I (as shown in FIG. 1), N-ibu-5'-O-DMT-deoxyguanosine-H-phosphonate, and 5'-HO-thymidine-3'-Olev were mixed together in pyridine and CD_3CN, as shown in FIG. 2. The reaction was followed by ^{31}P NMR for the formation of new phosphorus peaks, indicating formation of H-phosphonate diester linkages. When two peaks were observed indicating the formation of H-phosphonate diester linkages had formed, elemental sulfur powder was added to the reaction tube. After one hour, the product was again evaluated by NMR spectra measurement. The result of this analysis showed formation of phosphorothioated diester. These results demonstrate that a compound represented by Structural Formula I is effective at promoting condensation of a nucleoside and a nucleoside-H-phosphonate monomer.

EXAMPLE 2

Three different oligonucleotide dimers were synthesized utilizing a compound represented by Structural Formula I (as shown in FIG. 1) as the coupling reagent, as shown in the reaction scheme of FIG. 2. Two of the dimers were made by combining N-ibu-5'-O-DMT-deoxyguanosine-H-phosphonate (0.25M) in dry pyridine containing 10% N-methylimidazole (by volume) with either 5'-HO-deoxycytidine-3'-O-Lev or 5'-HO-deoxyadenosine-3'-O-Lev (1.1 equivalents relative to H-phosphonate). The third dimer

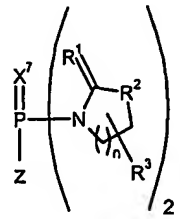
was generated by combining N-ibu-5'-O-DMT-thymidime-H-phosphonate with 5'-HO-deoxyguanosine-3'-O-Lev (1.1 equivalents relative to H-phosphonate). These condensation reactions were carried out by adding the coupling reagent a compound represented by Structural Formula I (1.5 equivalents with respect to H-phosphonate) in pyridine (4.0 ml/mmol), and was catalyzed by N-methylimidazole (10% of the solvent) at ambient temperature. The coupling reaction was carried out for approximately 20 to 30 minutes. The reaction product was then assessed by TLC and ^{31}P-NMR to determine when the H-phosphonate diester linkage had formed. Next, the sulfur transfer agent 3-(1,3-dioxo-1,3-dihydro-isoindol-2-ylsulfanyl)-propionitrile (hereinafter "CESP") (2 equivalents with respect to H-phosphonate) was added to the reaction mixture, and the reaction was carried out for 15 to 20 minutes at ambient temperature. The dimers DMT-O-dG(N-ibu)-PO-(SCH_2CH_2CN)-O-dC(Nbz)-3'-O-Lev (FIG. 3A), DMT-O-dG(N-ibu)-PO-(SCH_2CH_2CN)-O-dA(Nbz)-3'-O-Lev (FIG. 3B), and DMT-O-T-PO-(SCH_2CH_2CN)-O-dG(N-ibu)-3'-O-Lev (FIG. 3C), were successfully generated, with isolated yields of approximately 85 to 90%.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

CLAIMS

What is claimed is:

1. A method of preparing an H-phosphonate diester, said method comprising the coupling of an H-phosphonate with a substrate comprising a free hydroxy group in the presence of a coupling agent represented by the following structural formula:



wherein:

X^7 is =O or =S;

Z is a halogen;

R^1 is =O or =S;

R^2 is -O- or -S-;

R^3 is H or a substituent, preferably a halogen, a nitro group, or an alkyl group; and

n is 1 or 2.

2. The method of Claim 1, wherein the H-phosphonate is a protected nucleoside or oligonucleotide comprising a 3'-H-phosphonate function.

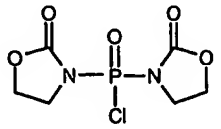
3. The method of Claim 2, wherein the nucleoside or oligonucleotide comprising a 3'-H-phosphonate function is selected from the group consisting of a ribonucleoside, a 2'-deoxyribonucleoside, a 2'-O-(alkyl, alkoxyalkyl or alkenyl)-ribonucleoside, an oligoribonucleotide, an oligo (2'-deoxyribonucleotide), and a 2'-O-(alkyl, alkoxyalkyl or alkenyl)-oligoribonucleotide.

4. The method of any one of Claims 1 to 3, wherein the substrate comprising a free hydroxy group is a protected nucleoside or oligonucleotide comprising a free 5'-hydroxy function.

5. The method of Claim 4, wherein the nucleoside or oligonucleotide comprising a free 5'-hydroxy function is selected from the group consisting of a ribonucleoside, a 2'-deoxyribonucleoside, a 2'-O-(alkyl, alkoxyalkyl, or alkenyl)-ribonucleoside, an

oligoribonucleotide, an oligo (2'-deoxyribonucleotide), and a 2'-O-(alkyl, alkoxyalkyl, or alkenyl)-oligoribonucleotide.

6. The method of any preceding claim, wherein the coupling agent is represented by the structural formula:



7. The method of any one of Claims 2 to 5, further comprising the steps of:

a. deprotecting a 5'-hydroxy group of the newly formed H-phosphate diester; and

b. optionally, repeating the coupling step and deprotection step one or more times, thereby forming an oligonucleotide.

8. The method of Claim 7, further comprising reacting the H-phosphonate diester with a sulfur transfer agent, thereby forming a phosphorothioate triester.

9. The method of Claim 8, wherein the sulfur transfer agent has the general chemical formula:

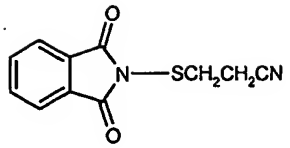
L-----S------A

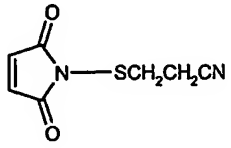
wherein L represents a leaving group, and A represents a methyl group, a substituted or unsubstituted alkyl group, or a substituted or unsubstituted alkenyl group.

10. The method of Claim 9, wherein the leaving group is a morpholine-3,5-dione, phthalimide, succinimide, maleimide or indazole, and A represents a 4- halophenyl group, 4-alkylphenyl group, methyl group, benzyl group, alkylbenzyl group, halobenzyl group, allyl group, crotyl group, 2-cyanoethyl group, or a 2-(4-nitrophenyl)ethyl group.

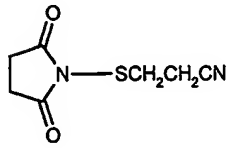
11. The method of Claim 9 or 10, wherein the sulfur transfer reagent is present during the coupling reaction and the phosphorothioate triester is formed *in situ*.

12. The method of Claim 9, 10 or 11, wherein the sulfur transfer reagent is one of the following compounds:



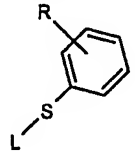


or



13. The method of Claim 12, wherein the phosphorothiate triester formed is reacted with a base to form a phosphorothioate diester.

14. The method of Claim 9, 10 or 11, wherein the sulfur transfer reagent is represented by the following structural formula:



wherein:

L is a leaving group; and

R is -H, an alkyl, or a halogen.

15. The method of Claim 14, wherein the phosphorothioate triester formed is reacted with an oximate to form a phosphodiester.

16. The method of Claim 7, further comprising the step of oxidative derivatization of the H-phosphonate backbone of the oligonucleotide after the oligonucleotide has been completely synthesized.

17. The method of Claim 16, wherein derivatizing the H-phosphonate oligonucleotide backbone comprises contacting the newly formed H-phosphonate diester with an oxidizing agent, a compound represented by R^{12}-X^6-H, and optionally, a base tertiary amine, wherein:

R^{12} is a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted aralkyl group; and

X^6 is -O-, -S-, or $-NR^{10}-$, wherein R^{10} is H, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted aralkyl group; or

X^6 is $-NR^{10}-$ and R^{12} and R^{10} taken together with the nitrogen to which they are attached form a heterocycloalkyl.

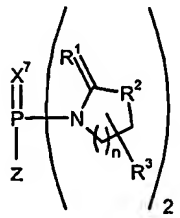
18. The method of Claim 16, wherein derivatizing the oligonucleotide backbone comprises contacting the newly formed H-phosphonate diester with an oxidizing agent, a tertiary amine, and water, thereby forming a phosphodiester.

19. The method of Claim 16, wherein derivatizing the oligonucleotide backbone comprises contacting the newly formed H-phosphonate diester with elemental sulfur and a tertiary amine, thereby forming a phosphorothioate oligonucleotide.

20. The method of any preceding claim, wherein the reaction is carried out in solution.

21. The method of any preceding claim, wherein the reaction is carried out on a solid support.

22. A method for producing an oligonucleotide H-phosphonate, comprising reacting an oligonucleotide comprising a free hydroxy function, with a substituted and unsubstituted alkyl H-phosphonate salt or a substituted or unsubstituted aryl H-phosphonate salt in the presence of a coupling agent represented by the following structural formula:



wherein:

X^7 is =O or =S;

Z is a halogen;

R^1 is =O or =S;

R^2 is -O- or -S-;

R^3 is H or a substituent, preferably a halogen, a nitro group, or an alkyl group; and

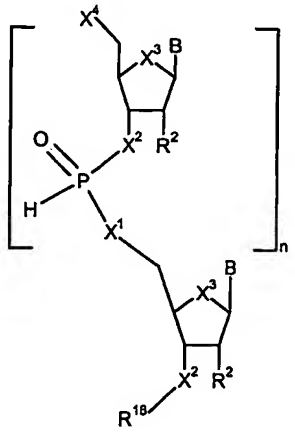
n is 1 or 2.

23. The method of Claim 22, wherein the oligonucleotide comprising a free hydroxy function has a free 3' or 5' hydroxy function.

24. The method of Claim 22 or 23, wherein the oligonucleotide is a protected oligo (2'-deoxyribonucleotide).

25. The method of Claim 22, 23 or 24, wherein the H-phosphate salt is an ammonium salt of a phenyl, alkylphenyl, or halophenyl H-phosphonate.

26. A method of condensing a nucleoside or a nascent oligonucleotide represented by the following structural formula:



wherein:

each X^1 is, independently, -O- or -S-;

each X^2 is, independently, -O-, -S-, CH_2, or $-NR^{11}-$;

each X^3 is, independently, -O-, -S-, $-CH_2-$, or $-(CH_2)_2-$;

X^4 is -OH or -SH;

R^2 is -H, -F, $-OR^6$, $-NR^7R^8$, or $-SR^9$;

R^{11} is -H, an alkyl group, an aryl group, or an aralkyl group;

R^6 is -H, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, a substituted or unsubstituted aralkyl group, or a hydroxy protecting group;

R^7 and R^8 are each, independently, -H, a substituted or unsubstituted aliphatic group, or an amine protecting group;

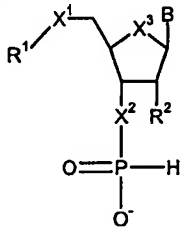
R^9 is -H, a substituted or unsubstituted aliphatic group, or a thio protecting group;

R^{16} is a hydroxy protecting group, a thio protecting group, an amino protecting group, a solid support, or a cleavable linker attached to a solid support, such as a group of the formula $-Y^2-L-Y^2-R^{15}$, wherein Y^2 for each occurrence is, independently, a single bond, -C(O)-, $-C(O)NR^{17}$-, -C(O)O-, $-NR^{17}$-, or -O-, wherein R^{17} is -H, a substituted or unsubstituted aliphatic group, or a substituted or unsubstituted aromatic group;

L is a linker; and R^{15} is a solid support;

each B is, independently, H or a protected or unprotected nucleoside base; and

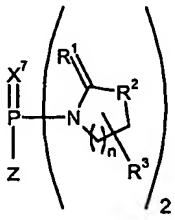
n is zero or a positive integer, with a salt of a monomer represented by the following formula:



wherein:

R^1 is an alcohol protecting group or a thio protecting group;

comprising the step of reacting the nucleoside or nascent oligonucleotide with the monomer salt in the presence of a coupling agent represented by the following structural formula:



wherein:

X^7 is =O or =S;

Z is a halogen;

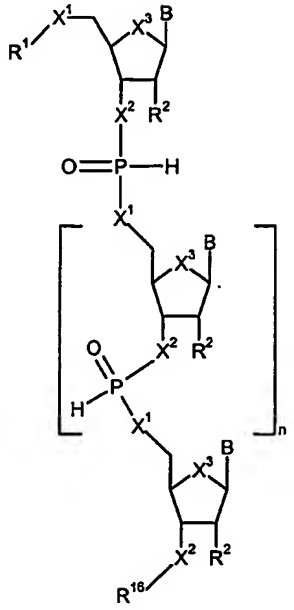
R^1 is =O or =S;

R^2 is -O- or -S-;

R^3 is H or a substituent, preferably a halogen, a nitro group, or an alkyl group; and

n is 1 or 2,

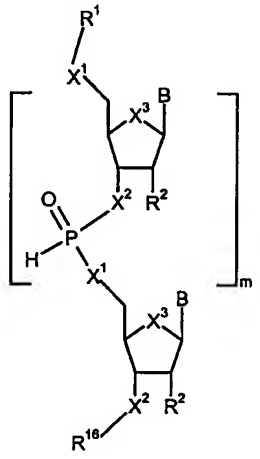
thereby forming a nascent H-phosphonate (n+1) oligonucleotide represented by the following structural formula:



27. The method of Claim 26, further comprising the steps of:

a. deprotecting the nascent (n+1) oligonucleotide by reacting it with a reagent to remove R^1; and

b. optionally repeating the coupling step and deprotection step one or more times to form an H-phosphonate oligonucleotide represented by the following structural formula:



wherein:

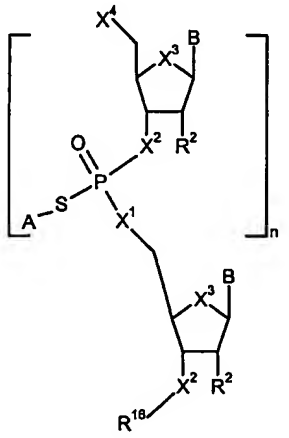
m is a positive integer.

28. The method of Claim 27, further comprising the step of reacting the fully synthesized H-phosphonate oligonucleotide with a sulfur transfer agent represented by the following structural formula:

L------S------A

wherein L represents a leaving group, and A represents a substituted or unsubstituted aryl group, a substituted or unsubstituted alkyl group, or a substituted or unsubstituted alkenyl group.

29. A method of condensing a nucleoside or a nascent oligonucleotide represented by the following structural formula:



wherein:

A represents a substituted or unsubstituted aryl group, a substituted or unsubstituted alkyl group, or a substituted or unsubstituted alkenyl group;

each X^1 is, independently, -O- or -S-;

each X^2 is, independently, -O-, -S-, CH_2, or $-NR^{11}$-;

each X^3 is, independently, -O-, -S-, $-CH_2-$, or $-(CH_2)_2-$;

X^4 is -OH or -SH;

R^2 is -H, -F $-OR^6$, $-NR^7R^8$, or $-SR^9$;

R^{11} is -H, an alkyl group, an aryl group, or an aralkyl group;

R^6 is -H, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, a substituted or unsubstituted aralkyl group, or a hydroxy protecting group;

R^7 and R^8 are each, independently, -H, a substituted or unsubstituted aliphatic group, or an amine protecting group;

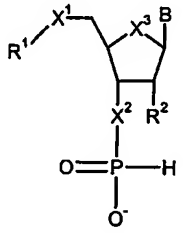
R^9 is -H, a substituted or unsubstituted aliphatic group, or a thio protecting group;

R^{16} is a hydroxy protecting group, a thio protecting group, an amino protecting group, or a solid support; or a cleavable linker attached to a solid support, such as a group of the formula $-Y^2-L-Y^2-R^{15}$, wherein Y^2 for each occurrence is, independently, a single bond, -C(O)-, $-C(O)NR^{17}$-, -C(O)O-, $-NR^{17}$-, or -O-, wherein R^{17} is -H, a substituted or unsubstituted aliphatic group, or a substituted or unsubstituted aromatic group;

L is a linker; and R^{15} is a solid support;

each B is, independently, H or a protected or unprotected nucleoside base; and

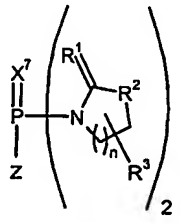
n is zero or a positive integer, with a salt of a monomer represented by the following formula:



wherein:

R^1 is an alcohol protecting group or a thio protecting group;

comprising the step of reacting the nucleoside or nascent oligonucleotide with the monomer salt in the presence of a coupling agent and a sulfur transfer agent, wherein the coupling agent is represented by the following structural formula:



wherein:

X^7 is =O or =S;

Z is a halogen;

R^1 is =O or =S;

R^2 is -O- or -S-;

R^3 is a H or a substituent, preferably halogen, a nitro group, or an alkyl group; and

n is 1 or 2,

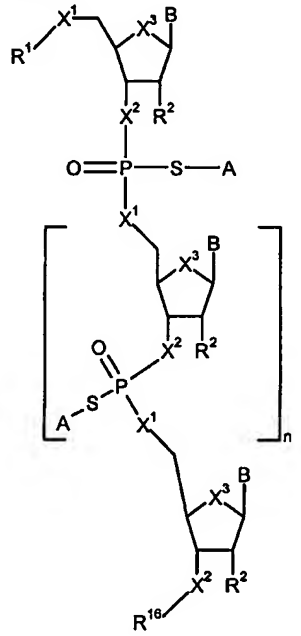
and the sulfur transfer agent is represented by the following structural formula:

L——S——-A

wherein:

L represents a leaving group and A represents a substituted or unsubstituted aryl group, a substituted or unsubstituted alkyl group, or a substituted or unsubstituted alkenyl group,

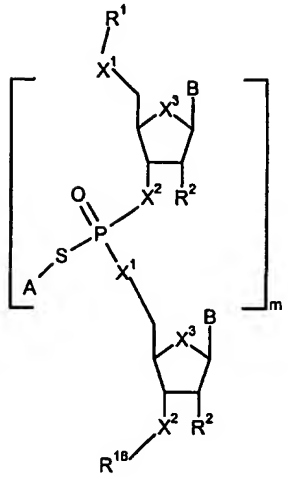
thereby forming a nascent H-phosphonate (n+1) oligonucleotide represented by the following structural formula:



30. The method of Claim 29, further comprising the steps of:

a. deprotecting the nascent (n+1) oligonucleotide by reacting it with a reagent to remove R^1; and

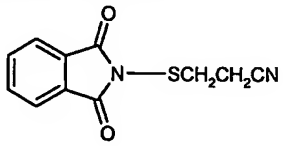
b. optionally repeating the coupling step and deprotection step one or more times to form a phosphorothioate triester oligonucleotide represented by the following structural formula:

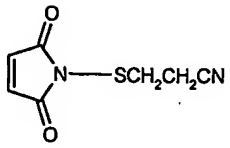


wherein m is a positive integer.

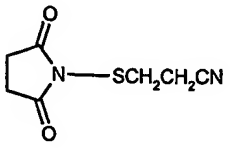
31. The method of Claim 26 or 29, wherein the leaving group is a morpholine-3,5-dione, phthalimide, succinimide, maleimide or indazole, and A represents a 4-halophenyl group, 4-alkylphenyl group, methyl group, benzyl group, alkylbenzyl group, halobenzyl group, allyl group, crotyl group, 2-cyanoethyl group, or a 2-(4-nitrophenyl)ethyl group.

32. The method of Claim 29 or 30, wherein the H-phosphonate oligonucleotide is contacted with one of the following compounds:

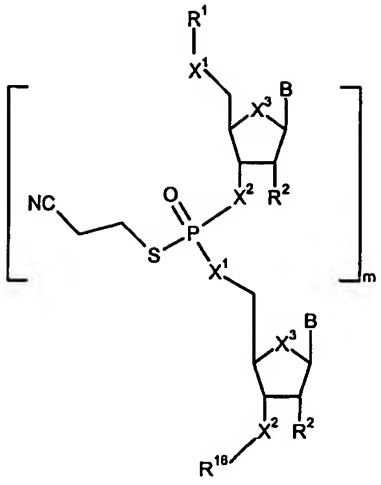




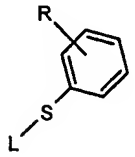
or



whereby the oligonucleotide formed is represented by the following structural formula:



33. The method of Claim 29 or 30, wherein the sulfur transfer reagent is a compound represented by the formula:



wherein:

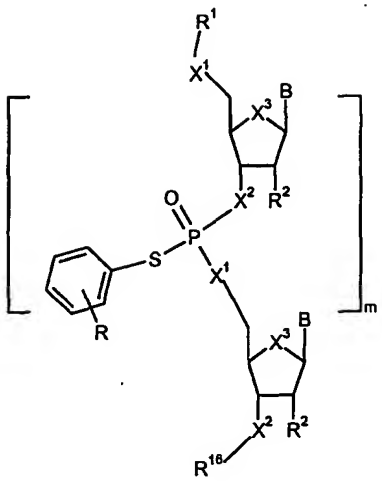
L is a leaving group; and

R is -H, an alkyl group, or a halogen,

and wherein the method further comprises the steps of:

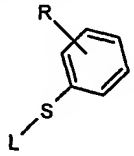
a. deprotecting the nascent (n+1) oligonucleotide by reacting it with a reagent to remove R^1; and

b. optionally repeating the coupling step and deprotection step one or more times to form a phosphorothioate triester oligonucleotide represented by the following structural formula:



and wherein after the oligonucleotide is fully synthesized, the backbone of the newly formed oligonucleotide is deprotected by contacting the oligonucleotide with an oximate and a base, thereby forming a phosphodiester oligonucleotide.

34. The method of Claim 29 or 30, wherein the H-phosphonate oligonucleotide is contacted with a compound represented by the formula:

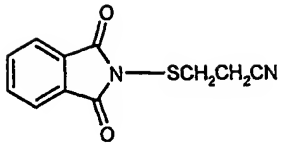


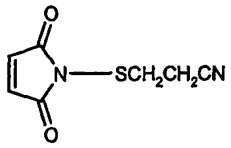
wherein:

L is a leaving group; and

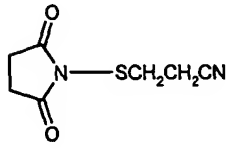
R is -H, an alkyl group, or a halogen,

after one or more condensation steps and is contacted with one of the following compounds:





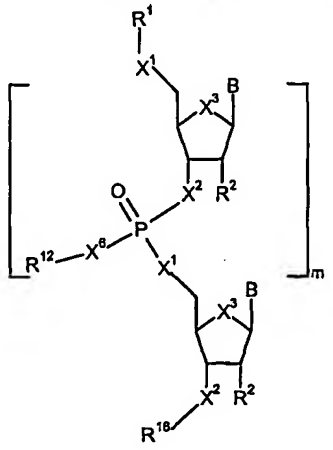
or



after one or more different coupling steps, and the phosphorothioate triester backbone of the oligonucleotide is deprotected by contacting the oligonucleotide with an oximate and a base, thereby forming a chimeric oligonucleotide.

35. The method of Claim 26, further comprising the step of oxidative derivatization of the H-phosphonate oligonucleotide backbone after the oligonucleotide is completely synthesized.

36. The method of Claim 35, wherein derivatizing the H-phosphonate oligonucleotide backbone comprises contacting the H-phosphonate oligonucleotide with an oxidizing agent, a compound represented by R^{12}-X^6-H, and optionally, a base tertiary amine, thereby forming an oligonucleotide represented by the following structural formula:



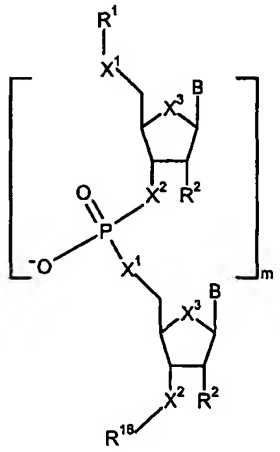
wherein:

R^{12} is a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted aralkyl group; and

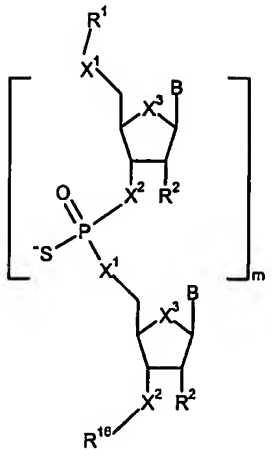
X^6 is -O-, -S-, or $-NR^{10}-$, wherein R^{10} is H, a substituted or unsubstituted aliphatic group, a substituted or unsubstituted aryl group, or a substituted or unsubstituted aralkyl group; or

X^6 is $-NR^{10}-$ and R^{12} and R^{10} taken together with the nitrogen to which they are attached form a heterocycloalkyl.

37. The method of Claim 35, wherein derivatizing the oligonucleotide backbone comprises contacting the H-phosphonate oligonucleotide with an oxidizing agent, a tertiary amine, and water, thereby forming an oligonucleotide represented by the following structural formula:



38. The method of Claim 35, wherein derivatizing the oligonucleotide backbone comprises contacting the H-phosphonate oligonucleotide with elemental sulfur and a tertiary amine, whereby the oligonucleotide formed is a phosphorothioate represented by the following structural formula:



39. The method of Claim 35, 36, 37 or 38, wherein derivatizing the oligonucleotide backbone is the final step.

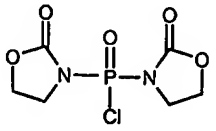
40. The method of Claim 35, 36, 37 or 38, wherein the deprotection step is the final step.

41. The method of Claim 26 or 29, wherein R^{16} is a cleavable linker of formula $-Y^2-L-Y^2-R^{15}$ or a solid support.

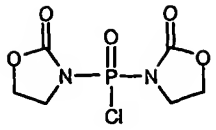
42. The method of Claim 26 or 29, wherein each R^2 is -OH or $-OR^6$ and the oligonucleotide prepared is a ribonucleotide.

43. The method of Claim 26 or 29, wherein each R^2 is -H and the oligonucleotide prepared is a deoxyribonucleotide.

44. The method of Claim 22, 26 or 29, wherein the coupling reagent is represented by the structural formula:



45. A process for the preparation of an oligonucleotide which comprises coupling a nucleoside or an oligonucleotide H-phosphonate monoester with a nucleoside or oligonucleotide comprising a free hydroxy group in the presence of a coupling agent, wherein the coupling agent is a compound of formula:



46. A process according to claim 45, wherein the oligonucleotide produced is an oligonucleotide phosphodiester, an oligonucleotide phosphorothioate, or a chimeric oligonculeotide comprising both phosphodiester and phosphorothioate moieties.

47. A process according to claim 45 or 46, wherein the oligonucleotide produced is a deprotected oligonucleotide.

48. A process according to claim 45 or 46, wherein the oligonucleotide produced is a protected oligonucleotide.

49. A process according to any one of claims 45 to 48, wherein a nucleoside 3'-H-phosphonate monoester is coupled with a nucleoside or oligonucleotide comprising a free 5'-hydroxy group.

FIG.1

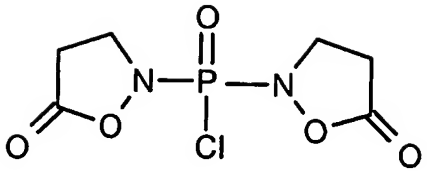
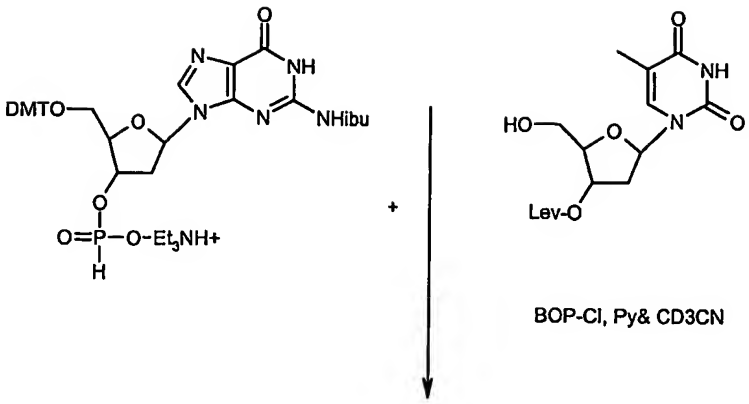


FIG. 2

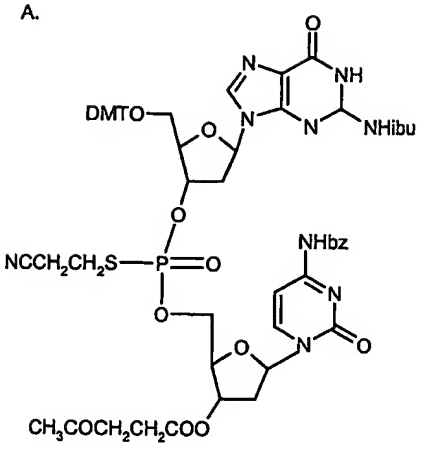


1. NMR spectra measurement showed formation of new phosphorus peaks

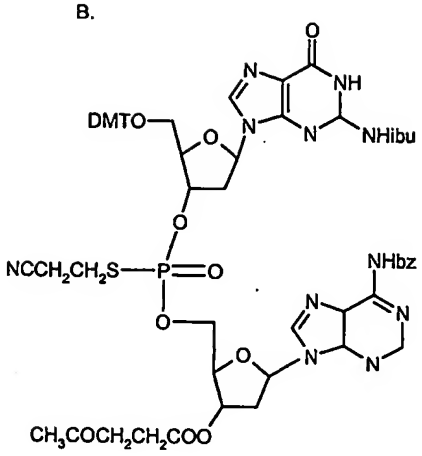
2. Elemental sulfur powder was added in the tube and spectra taken after 1hr showed the formation of Phosphorothioated diester.

FIGS. 3A-3C

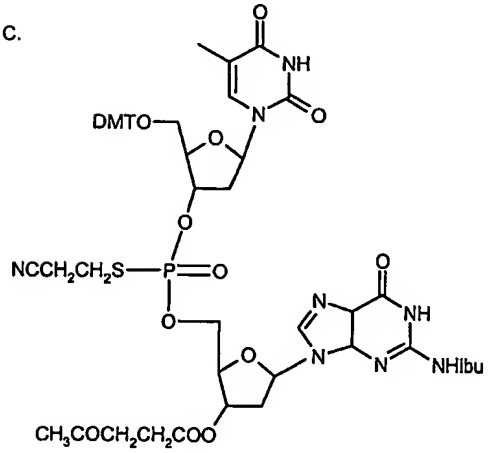
A.



B.



C.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/05177

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 207 819 B1 (MANOHARAN MUTHIAH ET AL) 27 March 2001 (2001-03-27) column 12, lines 28-29 examples 8-9 ---	1-8,19, 38-43
X	P.J. GAREGG ET AL.: "Nucleoside H-phosphonates. III. Chemical synthesis of oligodeoxyribonucleotides by the hydrogenphosphonate approach" TETRAHEDRON LETTERS, vol. 27, 1986, pages 4051-4054, XP002232291	1-7,16, 18-27, 35,37-49
Y	the whole document ---	8-15, 28-34
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents:

'A' document defining the general state of the art which is not considered to be of particular relevance

'E' earlier document but published on or after the international filing date

'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

'O' document referring to an oral disclosure, use, exhibition or other means

'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

'&' document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2003	11/03/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	de Nooy, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 723 973 A (KING S COLLEGE LONDON) 31 July 1996 (1996-07-31) compound 21 the whole document	8-15, 28-34
X	R. ZAIN, J. STAWINSKI: "Nucleoside H-phosphonates. 17. Synthetic and 31P NMR studies on the preparation of dinucleoside H-phosphonothioates" J. ORG. CHEM., vol. 61, 1996, pages 6617-6622, XP002232292	1-7,16, 18-27, 35,37-49
Y	the whole document	8-15, 28-34
Y	WO 01 64702 A (REESE COLIN BERNARD ;AVECIA LTD (GB)) 7 September 2001 (2001-09-07) the whole document	8-15, 28-34
X	KNERR L ET AL: "Efficient Synthesis of Hydrophilic Phosphodiester Derivatives of Lipophilic Alcohols via the Glycosyl Hydrogenphosphonate Method" TETRAHEDRON LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 39, no. 3-4, 15 January 1998 (1998-01-15), pages 273-274, XP004100940 ISSN: 0040-4039 the whole document	1,6
A	WO 99 09041 A (REESE COLIN BERNARD ;ZENECA LTD (GB); SONG QUANLAI (US)) 25 February 1999 (1999-02-25) cited in the application the whole document	1,8

INTERNATIONAL SEARCH REPORT

National application No.
PCT/GB 02/05177

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 17, 20-21 (in part), 36, 39-40 (in part)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 17, 20-21 (in part), 36, 39-40 (in part)

Present claims 17, 20-21 (in part), 36, 39-40 (in part) relate to an extremely large number of possible compounds for the derivatization of the H-phosphonate oligonucleotide. In fact, the claims contain so many options that a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Furthermore, the wording of claims 17 and 36 is unclear since it is ambiguous whether R12-X6-H is an oxidizing agent or whether it is not. Since there are no examples nor is there a further explanation in the description regarding this issue, there is also a lack of disclosure within the meaning of Article 5 PCT. Consequently, claims 17 and 36 have not been searched and those parts of claims 20-21, 39-40 relating thereto have not been searched either.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/05177

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		JP 2002536453 T	29-10-2002
		WO 0047593 A1	17-08-2000
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